Immunoreactivity of Protamine Preparations Used to Reverse Heparin Anticoagulation

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To determine if four commercially available intravenous protamine preparations differed in their ability to bind to human antiprotamine antibody, the sera of seven protamine-insulin-dependent diabetics who had experienced life-threatening reactions to intravenous protamine and whose sera contained a mean of 67.4 μg/ml (range, 16–200 μg/ml) of antiprotamine IgG antibody were evaluated. Each serum was preincubated with buffer and 0.0014 to 1.4 mg/ml of the four protamine preparations before addition to an agarose-based solid-phase radioimmunoassay using 125I-radioiodinated staph protein A as the detection protein. In the seven sera, the concentration of soluble protamine inhibiting 50% of protamine antibody (IC50) was determined by interpolating from points above and below 50% inhibition for each protamine preparation. No significant difference was found in the IC50 among the four different protamine preparations (P > 0.25; Kruskal-Wallis). The authors concluded that there is no significant difference in the immunoreactivity of the four commercial protamine preparations with human antiprotamine IgG antibody. Thus, there appears to be no advantage in using a particular intravenous protamine preparation based on immunoreactivity with human antiprotamine antibody. (Key words: Allergy, anaphylaxis; protamine. Blood, coagulation: heparin; protamine. Diabetes mellitus. Immune response.)

Protamine, which is used to reverse the anticoagulating effects of heparin, may cause several adverse reactions, including bronchospasm, pulmonary artery hypertension, and systemic hypotension.1 The exact etiology of these reactions remains unclear and appears to be multifactorial.2 Protamine is a foreign protein derived from fish sperm3 that can cause immunologic sensitization upon exposure in humans.2 This sensitization occurs most often in diabetic patients receiving insulin preparations containing protamine. Such protamine-insulin preparations include neutral protamine Hagedorn (NPH) and protamine zinc insulin (PZI). Others possibly at risk for sensitization are patients with previous exposure to intravenous protamine from its use following cardiac catheterization, cardiopulmonary bypass, vascular surgery, dialysis, or pheresis,4-5 and patients with histories of infertility6-9 or fish allergy,10 or in whom a vasectomy has been performed.

Previously, we showed that in diabetics taking insulin preparations containing protamine, the presence of both antiprotamine IgG or IgE was a significant risk factor for severe, life-threatening reactions when intravenous protamine was administered.11 It is possible that available intravenous protamine preparations obtained commercially differ in their ability to bind to human antiprotamine antibody. If a particular commercially available protamine is less immunoreactive with human antiprotamine antibody, then it may be expected to cause less frequent or less severe reactions and therefore would be more advantageous to use.

Because all antibodies produced against a specific antigen such as protamine contain two regions, the constant region (which determines the antibody class and biologic function) and the variable region (which is the antigen binding site of the molecule), and because all classes of immunoglobulins use the same set of variable region genes (defining the antigen binding site), when the class of immunoglobulin is changed, all that is switched is the constant region of the heavy chain.12 Therefore, all classes and subclasses of immunoglobulins directed against protamine contain the same variable region with identical antigen binding to protamine. This allows us the use of antiprotamine IgG antibody as a model of immunoreactivity for all classes of immunoglobulins, including IgE. This is advantageous because inhibition studies require 40–200 times less serum when evaluating antiprotamine IgG compared to antiprotamine IgE antibodies. We therefore compared the ability of four commercially available intravenous protamine preparations to bind antiprotamine antibody in the sera of diabetic patients who had experienced life-threatening reactions to intravenous protamine.

Materials

Following institutional approval, sera from seven protamine-insulin dependent diabetic patients who had experienced life-threatening reactions to intravenous prot-
amine were evaluated. We had previously determined that sera from all seven patients contained antiprotamine IgG antibody with a mean of 67 µg/ml (range, 16–200 µg/ml) of antiprotamine IgG antibody. Normal sera from healthy laboratory personnel with no history of protamine exposure or demonstrable antiprotamine antibodies were used to determine nonspecific binding in the protamine solid-phase radioimmunoassay (SPRIA). A high-titered, human, antiprotamine IgG serum containing 21.7 µg/ml of antiprotamine IgG antibody, which was quantitated using the technique of heterologous interpolation as previously described,\textsuperscript{13,14} was used to generate the standard curve.

ANTIGEN

Protamine sulfate preparations were obtained from the following pharmaceutical companies: Eli Lilly and Company (Indianapolis, IN), Quad Pharmaceuticals (Indianapolis, IN), Lyphomed, Inc. (Rosemont, IL), and Elkins-Sinn (Cherry Hill, NJ).

RADIOabeled DETECTION PROTEIN

The detection protein used was staph protein A. This protein has previously been shown to bind to human IgG 1, 2, and 4, which comprise approximately 93% total human IgG antibody.\textsuperscript{13} Staph protein A was radiolabeled with \textsuperscript{125}I by the chloramine T method, and the immunoreactivity was determined by trichloracetic acid precipitation.

PROTAMINE SORBENT

Protamine sulfate (Eli Lilly) was coupled to sepharose CL-4B beads (Pharmacia, Piscataway, NJ) after activation with cyanogen bromide as previously described.\textsuperscript{14}

Methods

Sera were preincubated with 5% sepharose CL-4B beads overnight to bind and remove naturally occurring antigastric IgG antibodies.\textsuperscript{15} Sera were evaluated in dilutions of 1:40 or greater (made RAST buffer [a phosphate-buffered saline containing 6.2% bovine serum albumin and 0.05% Tween 20]), and all dilutions beyond 1:40 were made in normal human serum diluted 1:40 to keep the quantity of total IgG antibody constant.\textsuperscript{15} All samples were studied in triplicate. One hundred microliters of sera were incubated with 100 µl of the four commercial protamine preparations for 1 h at room temperature at the following concentrations: 0.0014 mg/ml, 0.014 mg/ml, 0.14 mg/ml, and 1.4 mg/ml. Sera were also incubated with 100 µl of RAST buffer (control) for 1 h. Following incubation, 100 µl of this mixture was added to 500 µl of the protamine sorbent (1% v/v) and orbitally rotated for 4 h at room temperature. After three washes, followed each time by centrifugation (5 min, 2300 g), 500 µl of radiolabeled \textsuperscript{125}I staph protein A was added to the protamine solid-phase and rotated overnight at room temperature. Following four washes, the bound radioactivity was quantitated by gamma spectroscopy, and the amount of antiprotamine IgG antibody bound to the sorbent was interpolated from the dilution curve of the reference serum with known antibody content. This reference curve was mathematically fit by a three-parameter spline function as previously described.\textsuperscript{16}

Statistical analysis was performed using the Kruskal-Wallis test. The percent inhibition of antiprotamine IgG antibody binding to protamine agarose solid-phase was calculated using the following formula: percent inhibition = 1 – (antibody level with inhibition/antibody level with RAST buffer) × 100%.

Results

Soluble protamine competitively inhibited the binding of antiprotamine IgG antibodies to the protamine sorbent in a dose-dependent manner in all seven sera (fig. 1). The percent inhibition was minimal at soluble protamine doses of < 0.0014 mg/ml and nearly 100% at 1.4 mg/ml. The curves for the four commercial preparations were nearly superimposable (fig. 1). The specificity of the inhibition was confirmed by the inability of soluble protamine to inhibit the direct binding of antiragweed IgG antibodies to a ragweed-agarose sorbent (mean inhibition = 0%; n = 4; data not shown).

![Fig. 1. Protamine inhibition curves generated from sera (containing antibody to protamine) from seven patients preincubated with increasing concentrations of four (A-D) commercial preparations of protamine. Resulting inhibition of antiprotamine IgG antibody binding to protamine-agarose solid phase is plotted on the y axis (mean ± SEM).](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931352/ on 04/06/2017)
The concentration of soluble protamine causing 50% inhibition of antibody binding to the sorbent (IC\textsubscript{50}) was not different for the four protamine preparations (P > 0.25).

**Discussion**

Protamine neutralization of heparin has been associated with significant, catastrophic, adverse reactions. Several mechanisms have been postulated as the etiology of these reactions, including complement activation either through protamine-heparin complexes\textsuperscript{17-19} or through protamine-antiprotamine IgG interactions,\textsuperscript{20} thromboxane generation,\textsuperscript{21-23} inhibition of carboxypeptidase N,\textsuperscript{24} and immediate hypersensitivity reactions with antiprotamine IgE antibodies.\textsuperscript{11} It now appears that more than one mechanism is responsible for these reactions. Although the incidence of adverse protamine reactions remains low, there is now evidence of certain high-risk groups of patients who demonstrate an increased reaction frequency. Diabetic patients receiving protamine containing insulin preparations represent one such high-risk group, with several studies showing a 40- to 50-fold increased risk for life-threatening reactions when given intravenous protamine.\textsuperscript{25,26}

Prior immunologic sensitization to protamine is necessary for the production of protamine-specific antibody\textsuperscript{27} and may be responsible for the adverse protamine reactions seen in certain patient populations. This sensitization may occur from previous exposure to protamine following cardiac catheterization, cardiovascular surgery, dialysis or pheresis,\textsuperscript{4-6} autoimmunity to human protamine in men with previous vasectomies or a history of infertility,\textsuperscript{7-9} exposure during fish ingestion,\textsuperscript{10} and prior protamine-insulin usage.\textsuperscript{2,11,28,29} Antibodies to protamine may be fairly common in diabetic patients taking protamine-insulins (NPH or PZI). Using a double antibody, radioimmunoprecipitation assay, Kurtz et al. found that 91% of patients taking protamine-insulin preparations for 20 ± 10 yrs and 58% of patients receiving similar preparations for <1 yr developed IgG antibody to protamine.\textsuperscript{26} Nell et al., using an enzyme-linked immunosorbent assay, reported that 38% of NPH insulin-treated diabetics (on NPH insulin from 6 mos to 48 yr) had the antiprotamine IgG antibody.\textsuperscript{29} As we previously reported, the presence of antiprotamine IgG or IgE antibody was a significant risk factor for an acute cardiovascular reaction when intravenous protamine was administered.\textsuperscript{11} Protamine, however, is not a single chemical entity. Commercial protamine preparations are made from the sperm of salmon and related fish species.\textsuperscript{2} It is a mixture of simple proteins rich in arginine and highly basic, in which there are both intra- and interspecies differences.\textsuperscript{3} Therefore, we evaluated whether four commercial protamine preparations differed in their ability to recognize and combine with human antiprotamine antibody in sera obtained from seven protamine-insulin dependent diabetics.

The decision to evaluate antiprotamine IgG antibodies and the ability to extrapolate to antiprotamine IgE antibody binding are as follows: first, because serum contains ng/ml concentrations of antiprotamine IgE antibody and IgG antibodies are present in µg/ml concentrations, 40- to 200-fold less serum is required for IgG studies compared to that required for studies of IgE. This is a significant consideration when doing inhibition studies at multiple protamine concentrations in triplicate. Second, studies have proven that all classes of immunoglobulins (IgG, E, M, etc.) use the same set of variable region genes and hence have the same specificity (binding site) for antigen.\textsuperscript{12} Therefore, antiprotamine IgG and antiprotamine IgE antibodies have the exact same antigen binding region and the same immunoreactivity to protamine.

All seven patients had experienced a life-threatening reaction to intravenous protamine requiring vasopressor cardiovascular support. However, there was no significant difference in the immunoreactivity of the human antiprotamine antibody with the four protamine preparations (fig. 1). Based on similar immunoreactivity with human antiprotamine antibody, there is no advantage in choosing one particular commercially available protamine preparation over another.

**References**

anaphylactic reaction to protamine in a patient allergic to fish. ANESTHESIOLOGY 55:324–325, 1981


